INVESTIGATING THE ROLE OF p53 IN HERPES SIMPLEX VIRUS - 1 REPLICATION

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Abstract

Herpes simplex virus - 1 (HSV-1) is a common human double-stranded DNA virus that can cause a variety of pathologies, including recurrent oral lesions in the immunocompetent individual and fatal encephalitis in the immunocompromised individual or neonate. As there is currently no cure for HSV-1 infection, worldwide morbidity and mortality rates remain high. HSV-1 replication is under intricate control by both viral and cellular factors that dictate whether the virus undergoes productive lytic replication or enters a state of latency during which there is decreased viral gene expression and no virus production. The mechanisms that determine the fate of the virus are not completely understood. p53, the well-known tumor suppressor gene, is involved in various cellular responses to stress, such as viral infection. We hypothesized that p53 plays a role in the establishment of HSV-1 latency by negatively regulating HSV-1 replication through repression of viral gene expression via the ATM/ATR damage response pathway; we predicted that p53 accomplishes this by binding to the p53 responsive elements (RE) previously discovered in the HSV-1 genome. Viral yields were determined for HSV-1 strain KOS grown on HCT116 wild-type (p53 +/+) and HCT116 p53-deficient (p53 -/-) cells at a multiplicity of infection (MOI) of 1 and at 72 hours post-infection, with the prediction that the viral titer would be higher when the virus was harvested from the HCT116 p53-deficient cells. Our results demonstrate that there is no significant difference in HSV-1 titer between harvests from p53-deficient cells and wildtype cells under these conditions. This suggests that p53 does not play a vital role in promoting HSV-1 latency overall; rather, p53 may exert both positive and negative effects on HSV-1 replication at varying points in time without favoring one cycle over another. Future research, such as determining viral titers harvested from cells in which the levels of p53 have been increased by both overexpression and the use of pharmacological agents to stabilize endogenous p53 should be conducted to further elucidate these complexities.

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Introduction and Significance

Herpes simplex virus-1 (HSV-1) is a linear, double stranded DNA virus with a genome of 152 kbp encoding approximately 80 genes. [1] It is one of nine human herpesviruses that causes persistent infections with several pathologies, ranging from cold sores to encephalitis. [2,3] HSV-1 infection is common among humans and is associated with significant morbidity and mortality. [2,3] There is currently no known cure for HSV-1 infection.

HSV-1 is transmitted through direct contact with an infected individual, whereby the virus enters mucosal tissue and replicates. [2,3] Once the virus has completed initial infection, it resides in sensory nerve ganglia and enters latency. It is through this process that HSV-1 establishes persistent infection and evades cellular antiviral mechanisms. During latency, the viral genome is characterized by repressive viral heterochromatin and the expression of specific latency associated transcripts (LAT), which are thought to be promoted by low levels of ICP0. [4–6] In the presence of various cellular stressors, latency is disrupted and higher levels of ICP0 promote the establishment of euchromatin and renewed gene expression; it is by this mechanism that the reactivated virus undergoes a productive lytic replication cycle. [5] These processes are illustrated in Fig. 1. [6]

p53 is a tumor suppressor that is commonly mutated in most cancers and is involved in coordinating many cellular functions, including cell cycle arrest, the activation of apoptosis, and DNA repair. [7–9] The functions of p53 are intricately regulated by post-translational modification and protein-protein interactions. [10] The principal interacting partner of p53 is MDM2, which under normal conditions leads to the ubiquitination and proteasome-mediated degradation of p53. [11] This is made possible by the stabilization of MDM2 via the ubiquitin hydrolase known as HAUSP. [12] On the other hand, various stressors such as DNA damage via the ATM/ATR pathway, lead to phosphorylation of p53 and auto-ubiquitination of MDM2, which stabilizes p53. [13] Recently, p53 responsive elements (RE) have been discovered in the HSV-1 genome; because p53 exerts its function principally as a transcription factor through p53RE to activate or repress expression of target genes, this discovery suggests that p53 may be involved in activating or repressing HSV-1 replication via these p53RE. [9,14] The three p53RE identified in the HSV-1

genome appear to mediate repression of certain viral genes in a p53-dependent manner, including ICP4 and ICP8. [14] Based on these interactions, we hypothesized that p53 may play a major role in promoting HSV-1 latency via p53RE. Fig. 2 illustrates this idea.

While much progress has been made to understand the controls that dictate whether HSV-1 undergoes lytic replication or enters latency, much remains to be discovered to fully understand how HSV-1 interacts with the cell to define its fate. While previous studies have implicated p53 in HSV-1 replication, [15] the recent discovery of p53RE [14] has provided novel insight into how this important cellular factor may modulate virus replication. Hence, we hoped to support the idea that stabilization of p53 in response to ATR signaling represses viral replication via the p53REs, facilitating the establishment of latency, based on its apparently repressive effects on the essential HSV-1 ICP4 and ICP8 genes. [14] Having a better understanding of these mechanisms may reveal new therapeutic strategies aimed at p53 to prevent recurrent outbreaks and associated morbidities.

Fig. 1: Model for Epigenetic Regulation of HSV-1 [6]

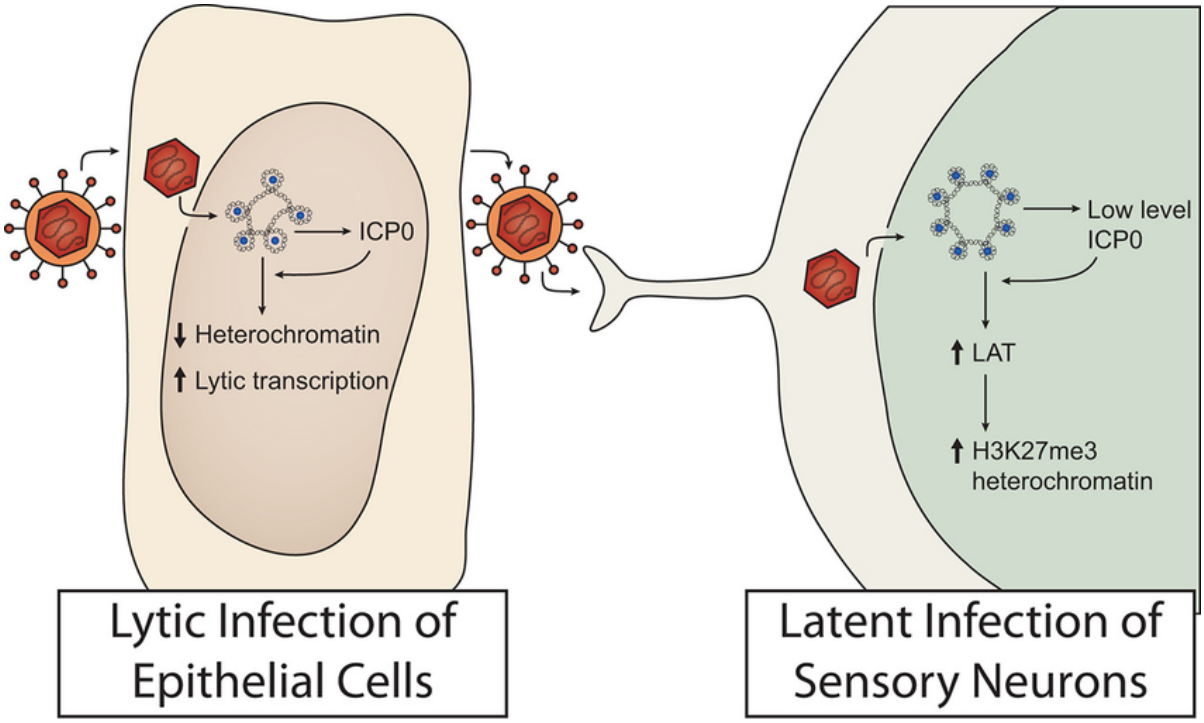


Fig. 1: Model for Epigenetic Regulation of HSV-1 [6] *This model, proposed by Raja et. al., illustrates how ICP0 is most likely involved in both the latent and lytic cycles of HSV-1. The lytic phase is characterized by decreased heterochromatin and increased euchromatin, while the latent phase is characterized by increased LATs and heterochromatin.*

Fig. 2: Model for the Role of p53 in HSV-1 Replication

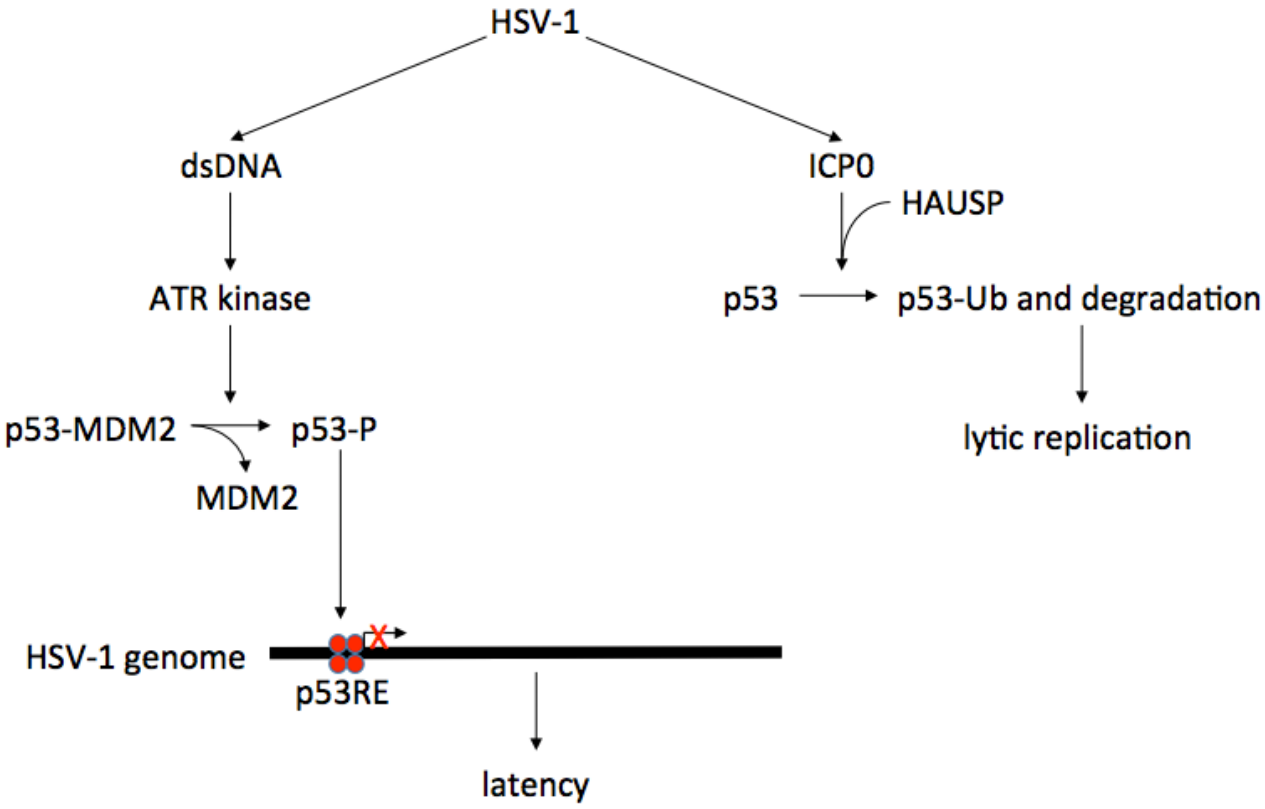


Fig. 2: Model for the Role of p53 in HSV-1 Replication *On the one hand, viral double-stranded DNA introduced during HSV-1 infection activates the ATR kinase DNA damage response pathway, leading to phosphorylation of p53 and dissociation of MDM2, thereby stabilizing p53 and allowing it to exert its effects via p53RE and establish latency. Conversely, higher levels of ICP0 in conjunction with the protease HAUSP and MDM2 stabilization lead to ubiquitination and degradation of p53; this prevents p53's repressive action and establishes a lytic replication cycle.*

Methods and Materials

Cell Culture and Maintenance

Two cell lines of human colorectal cancer origin, HCT116 wildtype (p53 +/+) (WT) and HCT116 knockout variants (p53 -/-) (KO), were acquired (a generous gift from B. Vogelstein). [16] They were grown in McCoy's 5A Medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S). The cells were incubated at 37°C in humidified air containing 5% CO_2 and passaged every 3-4 days. Since these human cells have previously been deidentified, our study was exempt from the University of Arizona IRB review.

Vero cells, which are African green monkey kidney fibroblasts that are a standard cell line for HSV-1 growth (ATCC), were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% glutamine, and 1% P/S. The cells were incubated at 37°C in humidified air containing 5% CO_2 and passaged every 2-3 days. These cells were grown in parallel and used as a control.

Viral Harvesting

At roughly 80-100% confluency, each of the three cell lines was infected with an HSV-1 KOS strain at a multiplicity of infection (MOI) of 1 (1 plaque-forming unit per cell). The viral solution was aspirated from the cells after 1 hour of infection time and the appropriate media was added for continued growth. At 72 hours post-infection, both supernatant and cell-associated progeny HSV-1 were harvested from each cell line and stored at -80℃.

Viral Titer Determination

The titers from each viral harvest were determined using a standard plaque assay protocol derived from several authors and optimized for our cell and virus types. [17,18] HSV-1 was serially diluted in serum-free DMEM by log10 to a suitable virion counting range, which was found to be between 10^{-7} to 10^{-5}. 100 μl of these viral dilutions were transferred to the confluent monolayers of Vero cells for a duration of one hour. The viral solution was then aspirated and the cells were fixed in equal parts 1.6% agarose and DMEM + FBS + P/S to allow for subsequent

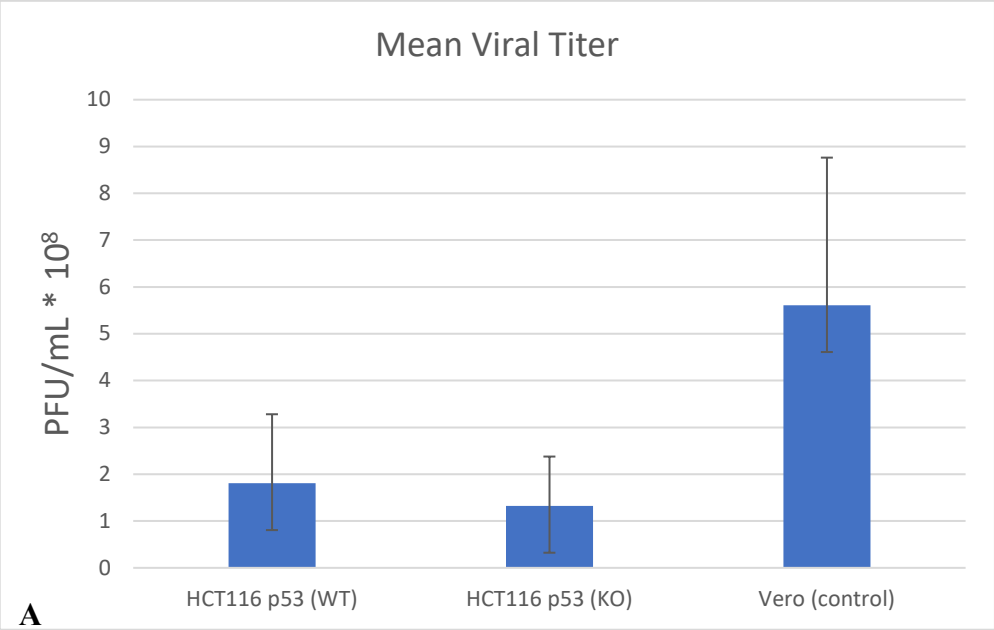
growth and plaque formation. After 5 days, the plaques were visualized using crystal-violet as a counter-stain. The plaques were counted using computer software that could track each plaque as it was counted. The assay was performed in duplicate during each trial; three trials were performed for a total of six experiments. The titers were averaged among the three trials to obtain precise values with standard deviations. Titers were determined using the following formula: [17,18]

$$titer = \frac{\#\ of\ plaques}{D\ x\ V}\ where\ D = dilution\ factor\ and\ V = volume\ of\ dilution\ added$$

Results

Our results demonstrate that at an MOI of 1 and a post-infection harvest interval of 72 hours, the mean titers of HSV-1 grown on either HCT116 p53(+/+) or HCT116 p53(-/-) cells were $1.8x10^8 \pm 1.5x10^8$ pfu/ml and $1.32x10^8 \pm 1.1x10^8$ pfu/ml, respectively (Fig. 3). There is no significant difference in these titers. As a control, the HSV-1 titer harvested from Vero cells was higher (mean $5.61x10^8 \pm 3.2x10^8$ pfu/ml) than that of the other two cell lines for each of the three trials.

Fig. 3: Titers of HSV-1 Harvested from HCT116 p53 (+/+) and p53 (-/-) Cells



Trial	HCT116 p53 (WT) *(* 10^8 PFU/mL)*	HCT116 p53 (KO) *(* 10^8 PFU/mL)*	Vero (control) *(* 10^8 PFU/mL)*
1	1.29	1.04	3.83
2	3.47	2.49	9.25
3	0.66	0.44	3.75
Average	1.81	1.32	5.61
B Stn. Dev.	1.47	1.05	3.15

Fig. 3: Titers of HSV-1 Harvested from HCT116 p53 (+/+) and p53 (-/-) Cells *Each cell line, HCT116 p53 WT and HCT116 p53 KO, was infected with HSV-1 at a multiplicity of infection (MOI) of 1. HSV-1 harvested from Vero cells was used as a control. The virus was harvested 72 hours post-infection. HSV-1 titers were assessed using standard plaque assays with Vero cells. Average titers are shown here. Standard deviations based on three trials performed in duplicate are shown (A). A table representing the average HSV-1 titers from each trial is displayed (B).*

Fig. 4: Representative Plaque Assay

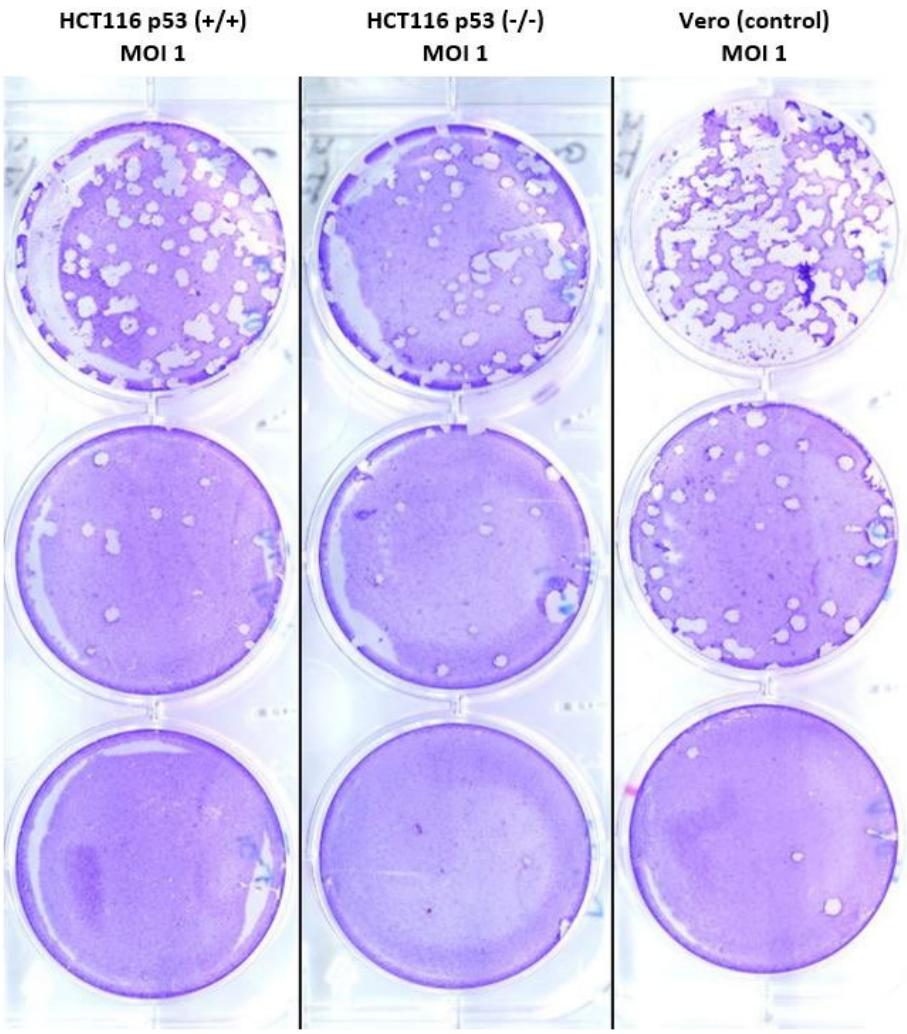


Figure 4: Representative Plaque Assay *Representative wells from Trial 1 are shown. At 72 hours post-infection, each well was stained with crystal violet for optimal plaque visualization. Viral titers were calculated as described under Methods and Materials.*

Discussion

There is some debate in the literature regarding whether p53 promotes or represses HSV-1 replication. In the paper by Hsieh et. al., it is argued that p53 represses the expression of HSV-1 proteins ICP4 and ICP8 via p53RE, suggesting that p53 represses HSV-1 replication and thereby shifts the virus into latency. [14] This is further supported by Hsieh's preliminary data showing reduced viral titers in the HCT116 p53 (+/+) cells compared to the HCT116 p53 (-/-) cells when the cells are infected with supernatant virus at an MOI of 10. In contrast, Maruzuru et al. suggests that p53 plays both positive and negative roles in HSV-1 replication but promotes it overall by upregulating ICP27 during early infection and downregulating ICP0 during later infection. [15] They performed plaque assays on Vero cells with HSV-1 harvested from HCT116 p53 (+/+) and HCT116 p53 (-/-) cells and found that at an MOI of 0.01 and at up to 18 hours post-infection, viral titers were reduced in the HCT116 p53 (-/-) cells compared to the HCT116 p53 (+/+) cells. [15] Our study measured titers under different conditions, with an MOI of 1 and a post-infection time of 72 hours prior to harvesting, and found no significant difference in viral titers between the different cell lines. The discrepancies in our results may support the idea that p53 exerts both positive and negative effects on viral replication and that its role may change depending upon the MOI, the number of hours post-infection prior to harvesting, and whether or not the supernatant virus, the cell-associated virus, or a combination are used to infect the cells.

One potential limitation of this study is the differing growth and metabolism rates among the HCT116 wild-type cells and the HCT116 p53-deficient cells. Though both cell lines are reported to have a doubling time of twenty-one hours, qualitative assessment demonstrated that the p53-deficient cells grew at a faster rate and had a higher metabolic rate as evidenced by the more rapid color change of the McCoy's 5A media. This is further supported by the concept that preventing a cell cycle regulating protein, such as p53, from being produced will enhance that cell's ability to proliferate and participate in metabolism. Though both cell lines were at a confluency of 80-90% at the time of HSV-1 infection, there could have been slight differences in

the confluency due to the differing growth rates and patterns, thus possibly influencing the MOI.

Another limitation was the differences in adherence properties among all three cell lines. Our original protocol proposed that we titer HSV-1 on HCT116 wild-type and HCT116 p53-deficient cells lines using a plaque assay to directly compare viral replication kinetics. Since our preliminary results demonstrated that the HCT116 cells did not adhere strongly enough to the plate surface and would peel off during the experiment, we decided to instead titer the virus on Vero cells only, with the changing variable being that the original virus used to infect the Vero cells was harvested from each of the three different cell lines. In addition, because Vero cells are extremely adherent to the culture plate surface, a cell scraper was occasionally used instead of trypsin to dislodge them when seeding a new plate. This introduced a higher degree of cell aggregation, which could have potentially skewed the results obtained during cell counting for

Future Directions

An important future experiment is to further analyze the dynamic replication kinetics of wild-type HSV-1. We intend to plot viral titer as a function of time, where the virus is harvested at intervals of 12 hours up to 72 hours post-infection. We also intend to compare the following cell lines: Vero, HCT116 p53 (-/-), HCT116 p53 (+/+), and HCT p53 (+/+) with overexpressed p53 using the pharmacological agent Nutlin. We will use Nutlin due to its properties as an MDM2 antagonist and its ability to thus stabilize endogenous p53. [19] We hope this will explain the discrepancies between our results and the results of both Hsieh et. al. and Maruzuru et. al. [14,15] Another interesting experiment would be to compare the viral titers of supernatant and cell-associated harvests, as these two harvesting conditions have been shown to have differing replication kinetics for wild-type HSV-1. [20] Measuring replication kinetics for HSV-1 mutants that lack the p53RE would also be useful, as the results would either support or refute the idea that p53 exerts its effects on HSV-1 via the REs previously identified.

Conclusions

Our results suggest that under our experimental conditions, p53 does not play a vital role in promoting latency overall; rather, p53 may exert both positive and negative effects on HSV-1 replication at varying points in time without favoring one cycle over another. The replication kinetics of HSV-1 in HCT116 p53 (+/+) and HCT116 p53 (-/-) cells may differ depending upon intracellular levels of p53, MOI, hours post-infection, and harvesting supernatant virus vs. cell-

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